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FEASIBILITY AND MASS, OUTDOOR CULTIVATION OF GONYAULAX CATENELLA

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National Center for Urban and Industrial Health, Cincinnati, Ohio

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Feasibility of Mass, Outdoor Cultivation of Gonyaulax Catenella

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Feasibility of Mass, Outdoor Cultivation of **Gonyaulax Catenella**

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Gulf Coast Shellfish Sanitation Research Center

Dauphin Island, Alabama

JULY 15, 1966

Details of illustrations in this document may be better studied on microfiche.

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
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Bureau of Disease Prevention and Environmental Control
National Center for Urban and Industrial Health
Water Supply and Sea Resources Program

I

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II

Foreword

This report was initiated by the Gulf Coast Shellfish Sanitation Research Center, Dauphin Island, Ala. The study upon which this report is based was accomplished by the Marine Biology Unit Under Project Order No. A1–6–F4408–O1–F4–WW from the Department of the Army, Edgewood Arsenal, Md., from 1 December 1965 through 28 February 1966.

The work presented in this report is the result of the efforts of many research center personnel as well as personnel associated with other Federal, State, and local agencies. In particular, acknowledgment is made to Mr. James H. Rogers and his maintenance staff, including Mr. Clinton A. Collier, Mr. Edward E. Williams, and Mr. William G. Zirlott, for fabricating and maintaining many of the laboratory and outdoor cultural systems; to Mr. Maynard W. Presnell for providing helpful technical advice throughout the study; to Mr. John J. Miescier for providing a great deal of technical assistance; to Mrs. Lillie M. Mason and Mr. Abner C. Jones III, for carrying out many of the media preparation, glassware treatment, and decontamination phases of the study; to Dr. M. Jeanne Kahnke for conducting the sulfide determinations; and to Mr. Donald L. Pugh, Process Engineering Branch, Operations Section, Edgewood Arsenal, Md., for providing helpful guidance.

Special acknowledgment is made to Miss Karen A. Steidinger and Mr. Richard P. Saunders, Florida State Board of Conservation, Marine Laboratory, St. Petersburg, Fla., for kindly identifying and enumerating the preponderant contaminating micro-organisms encountered during the outdoor pond studies; and to Mr. William N. McKeever (Economy Electric Construction Co., Mobile, Ala.) for providing many helpful suggestions regarding the design of the outdoor pond's temperature control systems.

Temporarily assigned to the Gulf Coast Shellfish Sanitation Research Center, Dauphin Island, Ala., to participate in this study. Present address: Weapons Development and Engineering Lab, Chemical Process Laboratory, ATTN: SMUEA–WCP, Process Engineering Branch, Operations Engineering Section, Edgewood Arsenal, Md. 21010.

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Abstract

OBJECTIVE

The object of the study was to determine the feasibility of mass culturing *Gonyaulax catenella* outdoors, in an enriched sea water medium, utilizing natural sunlight, and controlling temperature and initial pH.

RESULTS

Under the conditions of this study, *G. catenella* was not successfully cultivated outdoors.

CONCLUSIONS

1. The feasibility of mass, outdoor cultivation of *G. catenella* could not be determined.
2. Difficulties encountered during the mass, outdoor cultivation of *G. catenella* have been described and discussed.
3. The complexity of mass, outdoor cultivation of *G. catenella* is compounded by a paucity of information on the precise growth requirements of this organism.

RECOMMENDATIONS

1. It is recommended that mass, outdoor cultivation of *G. catenella* not be undertaken until more complete information has been obtained as to the growth requirements of this organism.
2. It is recommended that controlled laboratory studies be undertaken in order to define more accurately the growth requirements of *G. catenella*. The following factors should be considered:
   - *a.* Effects of alternating light-dark illumination on growth of *G. catenella* with emphasis oriented toward illumination intensity-duration relationships.
   - *b.* Methods of reducing or eliminating contaminants from the culture medium.
   - *c.* Effects of sea water and culture medium composition on *G. catenella* growth with emphasis on sea water quality.
   - *d.* Effects of aeration and fluctuating pH on *G. catenella* growth with emphasis on determining whether control is necessary.
   - *e.* Studies oriented toward selecting strains of *G. catenella* that exhibit growth characteristics conducive to outdoor cultivation.
   - *f.* Need and limits of temperature control for outdoor cultivation of *G. catenella*.

Introduction

Reports of human illnesses and deaths resulting from the consumption of toxic shellfish harvested along the Pacific coast of North America date back to 1793 when one of Captain Vancouver's seamen died after eating roasted mussels gathered on what is now Vancouver Island, British Columbia (*1*).

Years later, after many chemical, physical, and biological investigations, the marine dinoflagellate, *Gonyaulax catenella*, was definitely shown to be a source of a toxin, that when concentrated in shellfish during feeding, can subsequently cause paralytic shellfish poisoning when ingested by humans (*2*). The toxin, paralytic shellfish poison, is one of the most potent toxins known (*3*). Its public health significance is described by Meyer (*4*) and McFarren et al. (*5*).

One of the major problems associated with many of the past investigations of the toxin and its relationship to shellfish and man has been the difficulty of obtaining sufficient quantities of toxic materials with which to work.

The initial demonstration that *G. catenella* was the source of the toxin present in California mussels, *Mytilus californianus*, was based on the bioassay of extracts of natural plankton populations and mussels exposed to fresh sea water containing these same micro-organisms (*2*). Later, Riegel et al. (*6*) found chemical similarities between the toxin produced by *G. catenella* and that present in mussels. They also stated that only under the most favorable conditions would large scale collection of naturally occurring *G. catenella* blooms provide enough materials for chemical study of the poison.

The purification and characterization of the toxin present in shellfish required tremendous amounts of material. The California mussel, because of its fluctuating toxicity levels, was only an occasional source of the toxin. Alaska butter clams, *Saxidomus giganteus*, retain a similar toxin in their siphons for long periods of time. Even though the butter clams provided a toxin source throughout the year, occasionally it was necessary to process up to 600 pounds of clam siphons (8 tons of clams) to obtain 1 gram of poison (*3*).

Recently, the purification and characterization of the toxin produced by *G. catenella* was accomplished using axenic cultures of this organism maintained in a chemically defined medium (*7, 8*). The cultures maintained by Burke et al. (*7*) exhibited a considerable variation in toxin production that was attributed to undefined environmental factors. Burke and coworkers had difficulty purifying the toxin because of low toxin yield per cell, and the toxin's high polarity, water solubility, and dialyzability.

In order to obtain sufficient quantities of the toxin produced by *G. catenella* for future chemical and biological studies it will be necessary to scaleup present mass culture methods.

The purpose of this investigation was to study the feasibility of successfully mass culturing *G. catenella* outdoors, in an enriched sea water medium, utilizing natural sunlight, and controlling temperature and initial pH.

Materials and Methods

LABORATORY CULTURES

Source

Unialgal seed cultures of *G. catenella*[1] were supplied by the U.S. Army Edgewood Arsenal, Md. From this seed stock, progressively larger cultures were maintained to provide the inocula for the following studies: (1) Outdoor pond cultivation; (2) suitability of local sea water as the culture medium base; (3) effect of pond construction materials on the growth of *G. catenella;* and (4) effect of alternating light-dark illumination on *G. catenella* growth.

Cultural Conditions

Cultures maintained in the laboratory for the above purposes were prepared in 16-oz. prescription bottles, 2.5-liter low-form flasks, and 5-gal. carboys at 15.5±1° C. (fig. 1). Continuous illumination was provided by two to four 40-watt, cool-white fluorescent lamps suspended approximately 8 inches above the culture flasks and having an intensity of from 500 to 700 ft.-c. (foot candles) at the culture surface. In some instances, the 5-gal. carboy cultures were aerated with water-saturated air that had been filtered through charcoal and glass wool.

[1] Originally isolated in 1949 from the Pacific Ocean (Monterey Bay) by Herman Sommer and Lucile Foster of the Hopper Foundation, University of California Medical Center, San Francisco. Identification confirmed, August 1966, by Dr. Enrique Balech, Casilla de Correo 64, Necochea, Argentina. Toxigenicity of this strain confirmed by mouse assay.

FIGURE 1.—Cultivation of 5-gal carboy cultures used for outdoor pond inoculation

Glassware Treatment

All culture flasks were washed in an ethyl alcohol-sodium hydroxide solution,[2] rinsed an equivalent of 10 times with tapwater, followed by five distilled water and five twice-distilled, demineralized water rinses. They were then plugged with cheesecloth-cotton plugs and autoclaved for 15 min. at 121° C.

Hard-to-clean glassware was first washed in a 40–60 percent dicoloid-F[3] acid solution followed by the above cleaning and sterilization procedures. Pipettes received the same cleaning treatment, but were sterilized in the hot air oven for 2 hr. at 180° C.

To prevent any possible contamination of local bodies of water with *G. catenella*, all used glassware was autoclaved, as outlined above, and its contents poured into a sand-shell filter placed in the ground.

Medium Preparation and Inoculation

Laboratory cultures were cultivated in the enriched sea water medium presented in table 1.

The sea water used for the enriched sea water medium was autoclaved for 15 min. at 121° C., allowed to cool at room temperature for 24 hr., then cooled to approximately 15° C. prior to enrichment.

[2] Ethyl alcohol-sodium hydroxide solution: NaOH—100g.; distilled water—500 ml.; ethyl alcohol 95 percent—500 ml.

[3] Dicoloid-F—milkstone remover—trade name of the Diversey Corp., Chicago, Ill.

Table 1.—Composition of enriched sea water propagation medium

KNO_3 [1]	mg.	100
$K_2HPO_4 \cdot 3H_2O$ [1]	mg.	13.103
$FeCl_3$ [1]	mg.	1
$Na_2SiO_3 \cdot 9H_2O$ [1]	mg.	0.012
Sterile sea water [2]	ml.	1,000
pH [3]		8.5–8.6

[1] Stock solutions prepared with twice distilled, demineralized, tapwater.

[2] Salinity range 21.3–36.3‰ (parts per thousand).

[3] pH of medium adjusted with 1N NaOH or 1N HCl.

Final preparation and inoculation of the medium were accomplished by the addition of the chemical stock solutions, adjustment of the pH, and the inoculation of the medium with a volume of inoculum equal to 10 percent of the volume of the fresh medium. When possible, only cultures having cell densities greater than 10,000 cells/ml. were used to provide the inoculum. Inocula having as few as 1,000 cells/ml. were used with success; however, peak population densities were delayed.

Cell Enumeration

Viable cell counts per unit volume were the criteria for the demonstration of growth. For counting purposes, 1 to 10 ml. of culture sample was withdrawn from the well-mixed culture with a sterile pipette or glass tube and fixed with a volume of 0.1 percent formalin equal to 10 percent of the sample size. One ml. of the fixed sample was then pipetted into a Sedgwick-Rafter counting cell and the viable cells enumerated by the strip count method described in "Standard Methods for the Examination of Water and Waste Water" (*9*). Each count was calculated from the number of cells observed in two strips at 100X.

Formalin fixation prior to counting resulted in some cell distortion, the separation of many cell chains, and, most important, the cessation of cell motility. As a result, after fixation, the determination of viability was based on the presence or absence of the rich golden color characteristic of viable *G. catenella* cells.

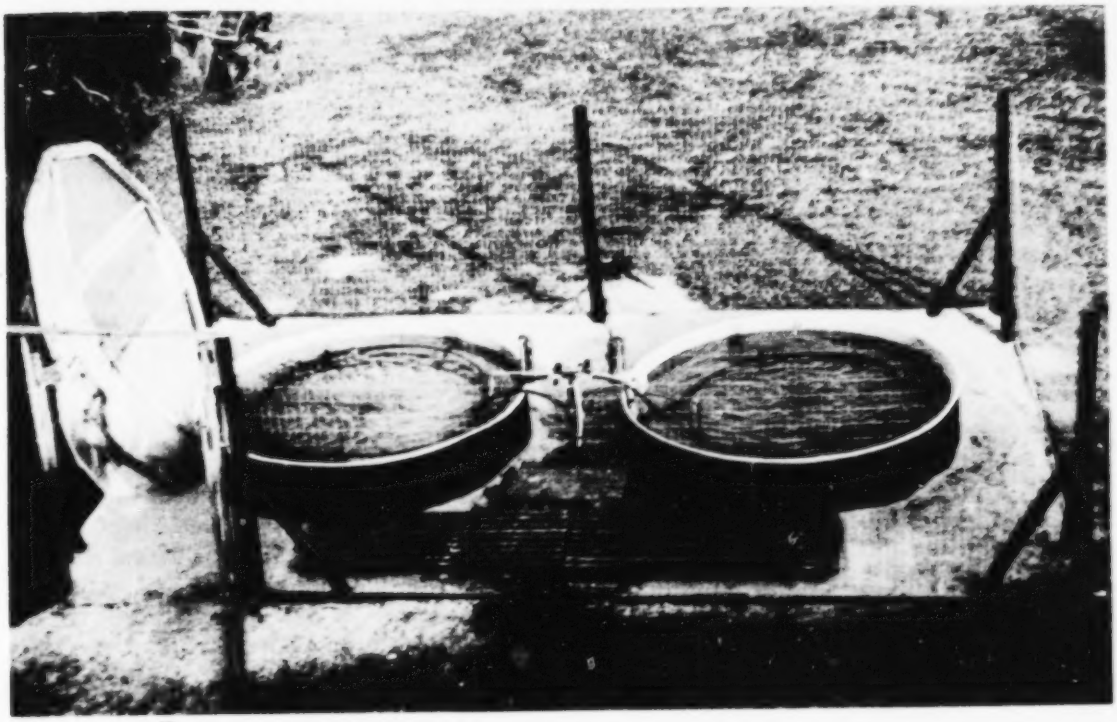

FIGURE 2.—Outdoor Culture Ponds

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Table 2.—Initial physical, chemical, and biological characteristics of pond studies 1, 2, 3, and 4

	Sea water base							Enriched sea water media [1]							
	Source	Date collected	Volume (liters)	Salinity (‰) [2]	pH	Total sulfides (mg./liter)	Treatment	Date	Temp (° C.)	Salinity (‰) [2]	pH	Inoculum		Initial *G. cutenella* cell count of pond culture (cells/ml.)	Culture depth (inches)
												Liters	Percent		
Pond study 1	Dauphin Island Bay.	1–22–66	1, 330	24. 1	8. 15	0. 006	Exposed to ultraviolet light. Stored in pond 4 days.	1–26–66	12. 4	24. 3	8. 65	148	11. 1	690	12. 3
Pond study 2	do	1–22–66	1, 330	21. 4	7. 95	. 004	Exposed to ultraviolet light. Stored in pond 4 days. 45# NaCl (crystals) added to increase salinity.	1–26–66	13. 9	34. 5	8. 55	148	11. 1	860	12. 3
Pond study 3	do	2–11–66	1, 400	28. 5			None	2–11–66	14. 0	28. 4	8. 65	148	10. 6	150	12. 8
Pond study 4	do	2–11–66	1, 400	28. 5			do	2–11–66	13. 9	28. 6	8. 60	0	0	0	11. 6

[1] Refer to table 3. [2] Parts per thousand.

OUTDOOR POND CULTURES

Cultural Conditions

The initial physical, chemical, and biological characteristics of pond studies 1, 2, 3, and 4 are presented in table 2.

Pond Systems

PONDS (Fig. 2)

The outdoor culture ponds consisted of two plastic swimming pools 8 ft. in diameter and 2 ft. deep installed on a level sand base. The pool liners were supported by a metal retaining wall and plastic clips—no part of which could come in contact with the culture medium. Drainage was provided through a rubber hose connected to the pool outlets and emptying into a sand-shell filter placed in the ground. During the pond studies, these outlets were capped with either a plastic cap or plugged with a black rubber stopper.

Convex, polyethylene covers were fabricated for each pond to provide coverage only in the event the success of a study was in jeopardy due to significant salinity reductions that would result from excessive rainfall. These covers were used during the media preparation phases of pond studies 1, 2, 3, and 4 and for a short time during the culture phase of pond study 3.

TEMPERATURE CONTROL

The winter air temperatures experienced on Dauphin Island necessitated the mechanical temperature control of the outdoor pond cultures as a means of continuously maintaining them at approximately 15° C.

During pond studies 1 and 2, each pond was equipped with a hot water temperature control system (fig. 3) designed to automatically compensate for the low air temperatures that would have otherwise lowered the culture temperatures well below 15° C. When the temperature of a culture fell below 15° C. a temperature controller pneumatically opened a modulating valve that allowed 66° C. water to be pumped through the heat exchange coils until the desired culture temperature (15° C.) was reached.

Daytime air temperatures experienced during pond studies 1 and 2 were not always low enough to compensate for the heating effect of the sun's radiant energy. This resulted in cultures rising above 15° C. with no means of cooling them. This problem, together with the hot water temperature control system's low sensitivity, resulted in the design of the more versatile hot and chilled water temperature control system used during pond studies 3 and 4 (fig. 4). Employing this system, the temperature of both ponds was controlled by two mercury thermoregulators placed in one "master" pond. One thermoregulator was connected to a normally closed, electronic relay that upon a decrease ($<15°$ C.) in culture temperature opened a normally closed, electric solenoid valve allowing 66° C. water to gravity feed through the heat exchange coils in both ponds until the temperature of the master pond rose to 15° C. The other thermoregulator was connected to a normally open, electronic relay that upon an increase ($>15°$ C.) in culture temperature opened a normally closed, electric solenoid valve allowing 8° C. water to gravity feed through the same heat-exchange coils until the temperature of the master pond decreased to 15° C. As a means of obtaining the heat exchange necessary for the efficient operation of the chilled water cycle, it was necessary to replace the thick-walled, black plastic coils with thin-walled, plastic coils having the dimensions described in figure. 4.

AERATION

Air supplied to the pond cultures to effect gentle agitation was charcoal-glass wool filtered prior to being distributed through a polyvinyl chloride distribution manifold. The rate of aeration and the occasional necessity of halting aeration to prevent an extreme rise in culture temperature precluded complete mixing of the culture.

Media Preparation and Inoculation

Based on the results of preliminary sea water suitability studies, relatively low salinity sea water from Dauphin Island Bay was used as the base for the enriched sea water media used in all four pond studies. The composition of the enriched sea water media is presented in table 3.

Although none of the pond studies were identical, the following sequence of pond preparation, media preparation, and inoculation was the same for each pond:

1. New pond liner installed and rinsed with sea water.

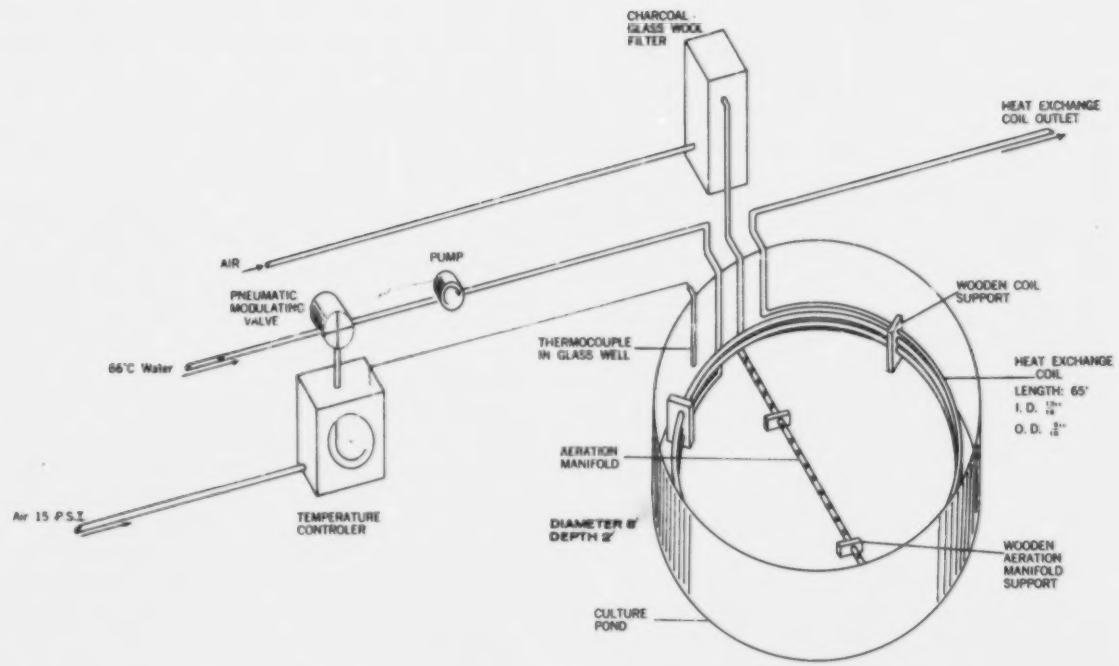

FIGURE 3.—"Hot Water" Temperature Control System (Pond Studies 1 and 2)

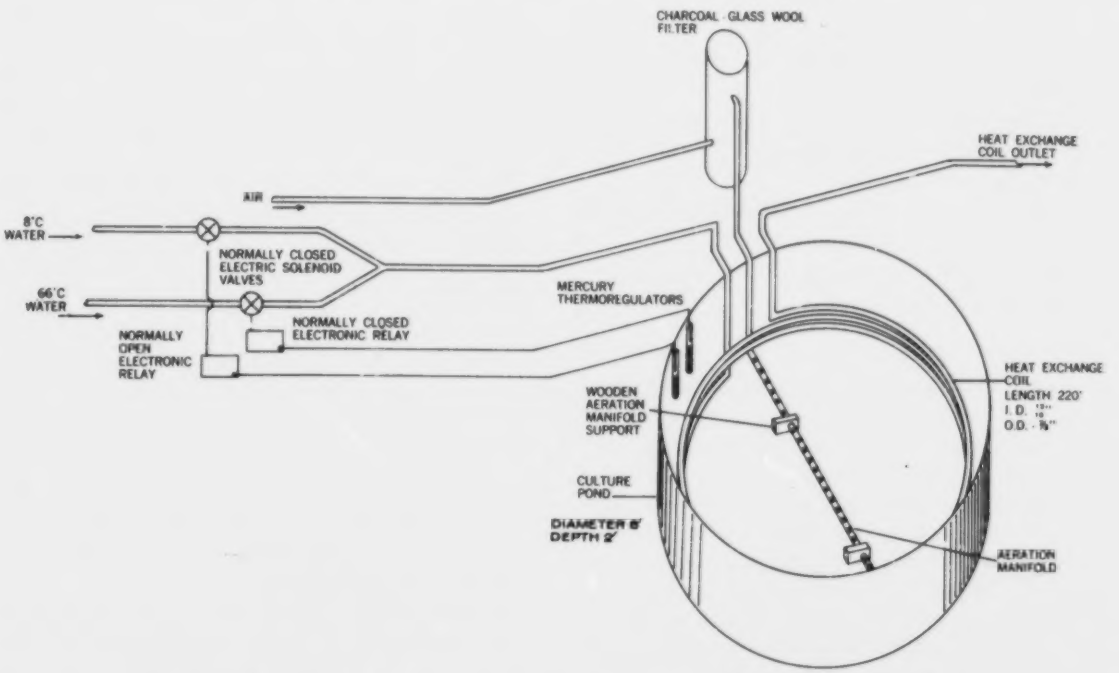

FIGURE 4.—"Hot and Chilled Water" Temperature Control System (Pond Studies 3 and 4)

Table 3.—Composition of enriched sea water propagation media

	Pond studies 1 and 2	Pond studies 3 and 4
KNO_3 [1]—mg.	100	100
$K_2HPO_4 \cdot 3H_2O$ [1]—mg.	13. 103	----------
K_2PO_4 [1]—mg.	----------	10
$FeCl_3$ [1]—mg.	1	1
$Na_2SiO_3 \cdot 9H_2O$ [1]—mg.	0. 012	130. 789
Raw sea water—ml.	[2] 1, 000	[3] 1, 000
pH [4]	8. 55–8. 65	8. 6–8. 65

[1] Stock solutions prepared with twice distilled, demineralized tapwater.
[2] Exposed to ultraviolet light prior to enrichment to reduce contamination. Pond 1—Salinity 23.5‰ (parts per thousand) (natural) prior to enrichment. Pond 2—Salinity 34.8‰ (artificial) 45# NaCl crystals added prior to enrichment to increase salinity from 21.4‰ (natural) to 34.8‰ (artificial).
[3] Not exposed to ultraviolet light prior to enrichment. Ponds 3 and 4—Salinity 28.5‰ (natural) prior to enrichment.
[4] pH of media adjusted with 1N NaOH.

2. Pond filled with sea water and the volume calculated using the mean depth and pond diameter.
3. Stock solutions of enrichment chemicals added in appropriate amounts and mixed by aeration.
4. pH adjusted with 1N NaOH.
5. Temperature of culture medium brought to inoculating temperature.
6. Culture medium inoculated with 5-gal. carboy cultures of *G. catenella* cultivated in the laboratory. (Note: Pond culture 4 was not inoculated with *G. catenella*.)
7. At termination of study, pond culture destroyed with 3 p.p.m. $CuSO_4 \cdot 5H_2O$ and passed through sandshell filter.
8. Aeration manifold and heat exchange coil removed prior to addition of $CuSO_4 \cdot 5H_2O$ and cleaned with sea water.
9. Used pond liner burned.

Sampling Procedure

During the pond studies, selected physical, chemical, and biological characteristics of the culture media and external environment were measured and recorded three times daily. Recorded each morning were the culture temperature, salinity, pH, *G. catenella* cell count, air temperature, light intensity, cloud coverage, and the inches of precipitation falling over a 24 hr.-period. With the exception of salinity, precipitation, and cell counts, each of these was also measured in the afternoon and evening. Sunrise-sunset information was recorded to indicate the total possible hours of sunlight available daily. The total sulfide concentration of the sea water used during pond studies 1 and 2 was determined immediately after the culture ponds were filled.

Culture temperatures were measured to the nearest 0.1° C. with a mercury thermometer. Air temperatures were continuously recorded by a Foxboro Dynalog temperature recorder.

Samples of culture were collected in sterile glass flasks for salinity and pH determinations which were made immediately after collection. Salinity was determined hydrometrically and reported as parts per thousand (‰) (*10*); a Beckman Zeromatic pH meter was used to measure the hydrogen ion concentration.

Light intensities were measured in foot candles using a General Electric light meter (type 213). The amount of cloud cover was estimated visually. Sunrise-sunset information was obtained from the "Table of Sunrise and Sunset at Mobile, Alabama" (*11*). Total sulfide concentrations were determined by the colorimetric method described in "Standard Methods for the Examination of Water and Wastewater" (*9*).

Culture samples used for the enumeration of *G. catenella* and other micro-organisms were withdrawn with sterile 10 ml. pipettes from five sampling sites in each pond. Four sites were evenly spaced around a pond, approximately 18 inches from the perimeter; one site was located in the center. Core samples were collected at each site by the slow immersion of the pipettes to the pond bottom.

During the first 5 days of pond studies 1 and 2, conducted simultaneously, culture sampling was conducted without prior mixing other than that supplied by aeration. To eliminate the effects of stratification due to the settling and eddying of *G. catenella* and other micro-organisms, the practice of thoroughly stirring the cultures prior to sampling was initiated.

Cell Enumeration

Except for the necessity of counting unfixed material near the end of pond study 3, the *G. catenella* fixation and counting procedures previously described were followed.

During pond studies 1 and 2, two cell counts were made on each of the five site samples. The mean daily cell count was calculated using the 10 resulting counts.

The mean daily *G. catenella* counts obtained during pond study 3 were calcu'ated from duplicate cell counts made on a composite sample of the five site samples. As a means of enumerating the very low *G. catenella* population existing near the end of this study, it was necessary to make counts on unfixed, rather than fixed materials.

Culture conditions present during pond studies 1 and 2 supported such dense populations of micro-organisms, other than *G. catenella*, that it was necessary to identify and enumerate these organisms during pond study 3 and particularly during pond study 4, which was conducted for the sole purpose of studying these contaminants.

Results

PRELIMINARY CULTURAL CONDITION EXPERIMENTS

Sea Water Suitability Studies

It was anticipated that low salinity sea water (20–25‰) would have to be used as the culture medium base. The expected occurrence of meteorologic and hydrographic conditions characteristic of the Dauphin Island area would severely hamper or prevent the collection of large volumes of high salinity sea water (30–35‰).

This problem prompted a series of sea water suitability studies in which the growth characteristics of *G. catenella* maintained under laboratory cultural conditions in media of naturally low, artificially high, and naturally high salinities were compared. The growth of *G. catenella* maintained in media in which the sea water base had been Millipore filtered (0.45μ), rather than autoclaved, was also observed.

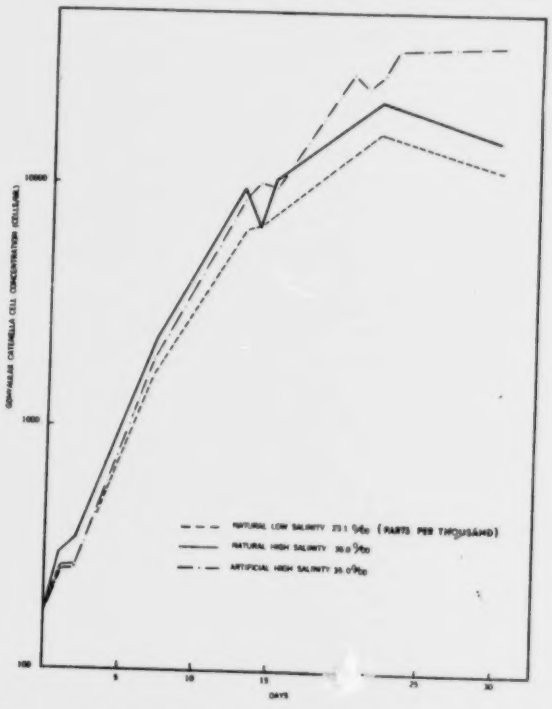

FIGURE 5.—Growth of Gonyaulax Catenella in Natural Low, Natural, Low, Natural High, and Artificial High Salinity Media (Millipore filtered)

The results of these studies demonstrated that *G. catenella* growth was supported by media having natural salinities as low as 21.3‰ as well as by media in which salinities were artificially increased to approximately 35‰ by the addition of sodium chloride crystals or "rock salt." The results of one of these studies are presented in figure 5. *G. catenella* growth in the medium in which salinity had been increased from 23.1‰ to 35‰ with sodium chloride paralleled the growth supported by the natural high salinity (35‰) and natural low salinity (23.1‰) media through the 15th day. By the 30th day of growth, the *G. catenella* density in the artificial high salinity medium exceeded that of both the natural high and low salinity media. Millipore filtration of the media's sea water base resulted in an extension of the growth phase of *G. catenella* that was not consistently observed in media in which the sea water base had been autoclaved. However, since it was not practical to Millipore filter the large volumes of sea water used throughout this investigation, all sea water used in the remaining studies was autoclaved.

Materials Compatibility Study

The design of the outdoor culture ponds and their environmental control systems called for the use of several construction materials (plastic pools, polyvinyl chloride pipe, black plastic pipe, wooden supports, and stainless steel hose clamps) whose effect on the growth or *G. catenella* was unknown.

A preliminary study was conducted to quickly determine which materials, when in continuous contact with the culture medium, were inhibitory to laboratory cultures of *G. catenella* cultivated under the laboratory cultural conditions previously described.

The results indicated that, with the exception of the stainless steel clamps, the other construction materials listed were compatible with *G. catenella* (fig. 6). However, since the retention of the

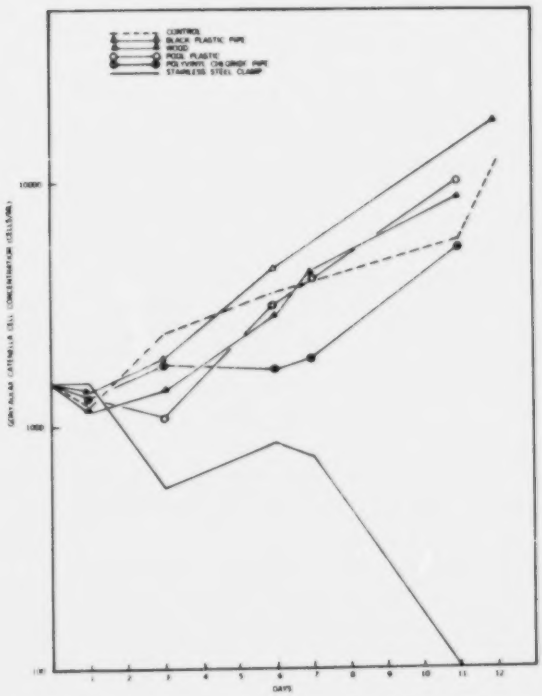

FIGURE 6.—Growth of Gonyaulax Catenella under continuous exposure to pond construction materials

clamps in the design of the outdoor ponds was necessary to provide the ponds with watertight connections, each clamp was well taped with black plastic electrical tape to prevent it from coming in contact with the culture media.

Illumination Studies

I. NATURAL ILLUMINATION VS. ARTIFICIAL CONTINUOUS ILLUMINATION

The effect of natural illumination, characterized by alternating light-dark conditions of relatively high light intensities, on *G. catenella* growth was the least understood of all the cultural conditions that were to be monitored during the outdoor pond studies. As a means of better understanding the role of illumination, a small study was conducted to determine the difference between the growth of *G. catenella* maintained under continuous, low intensity illumination in the laboratory and that of cultures maintained outdoors under alternating light-dark, relatively high intensity illumination.

Three 2.5-liter low-form flasks each were filled with 1 liter of culture medium. The medium's salinity was increased from 31.9‰ to 35.3‰ with the addition of sodium chloride crystals. Each flask was inoculated from the same stock culture. One flask was maintained under the laboratory conditions previously described. Two flasks were immersed to a depth of 1 inch in an outdoor pond and maintained at approximately 15° C. The mouths of the outdoor flask were covered to prevent rain from entering and lowering the salinity of the cultures. None of the cultures were aerated. Each culture was observed for 13 days before the outdoor cultures were placed under continuous illumination in the laboratory. The cultures were then observed for an additional 12 days.

The cultures maintained under continuous, low intensity illumination remained in the lag phase for 4 days and then proceeded to reach a maximum density of 14,000 cells/ml. after 16 days of growth (fig. 7). The population densities of both outdoor cultures exhibited an immediate decline followed by a leveling off at a low density after 5 days. This low density maintenance was interrupted by a marked increase in cell numbers after

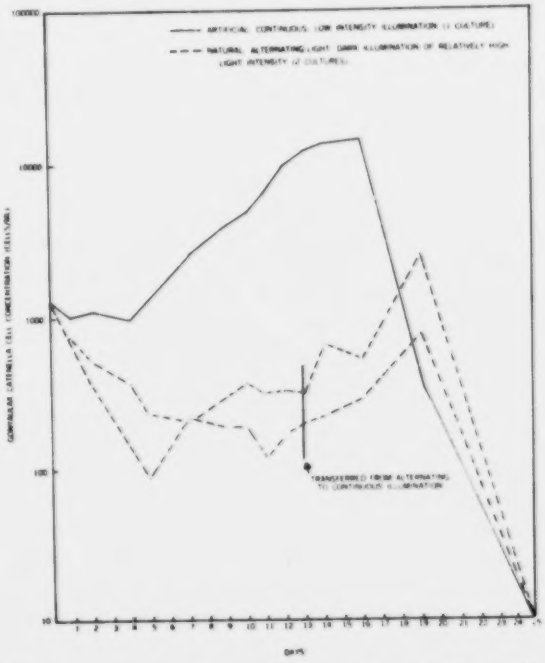

FIGURE 7.—Growth of Gonyaulax Catenella under artificial, continuous, low intensity illumination (700 ft-C) and natural, alternating light—dark illumination of relatively high light intensity (200–5,000 ft-Clave.: 10 hrs. 22 mins. light, 13 hrs. 38 mins. dark

the cultures were brought into the laboratory and subjected to continuous, low intensity illumination.

With the exception of the intensity and duration of illumination and a slight temperature variation, the cultural conditions of both the outdoor and laboratory cultures were comparable. Depending upon the attitude of the sun and the degree of cloud cover, the outdoor cultures received from 200–>5,000 ft.-c. of light for an average of 10 hr. 22 min. daily, whereas the laboratory culture received 700 ft.-c. of light continuously. The temperature of the laboratory culture was maintained at 15.5±1° C.; the temperature of the outdoor cultures averaged 14.9° C. and ranged from 12 to 19° C.

The satisfactory growth of the laboratory culture and the poor growth exhibited by the outdoor cultures until exposed to continuous, low intensity illuminating indicated that either: (1) high light intensities; (2) reduced periods of illumination; (3) fluctuating light intensities; or (4) a combination of these factors, coupled with minor temperature fluctuations, may have been responsible for the poor growth of the outdoor cultures under natural illumination.

II. ARTIFICIAL ALTERNATING LIGHT-DARK ILLUMINATION VS. ARTIFICIAL CONTINUOUS ILLUMINATION

The effect of alterating light-dark illumination on the growth of *G. catenella* was investigated further during pond studies 1 and 2.

Immediately after pond inoculation, two subculture samples were withdrawn from each pond and placed in 2.5-liter low-form flasks. One sample was cultured at 15.5±1° C. under continuous, low intensity illumination (700 ft.-c.); the other sample was cultured at 14.5° to 21.5° C. under alternating (11 hr. light-13 hr. dark) light-dark illumination (700 ft.-c.). No aeration was provided. These cultural conditions were maintained through the eighth day, after which the subcultures exposed to alternating light-dark illumination were placed under continuous illumination. The study was terminated on the 13th day.

The subcultures from pond studies 1 and 2, maintained under continuous illumination, attained higher *G. catenella* population densities that began their decline sooner than did those subcultures initially exposed to alternating illumination and later exposed to continuous illumination

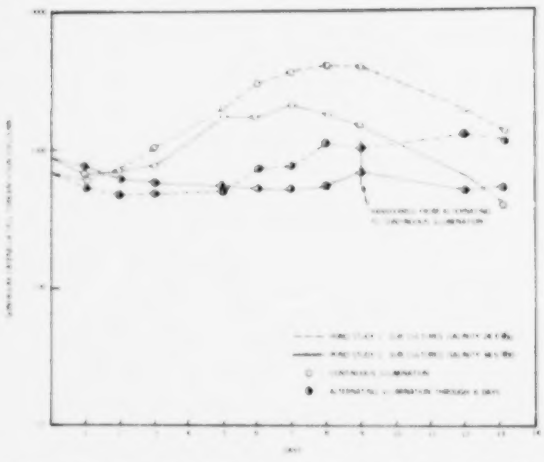

FIGURE 8.—Growth of Gonyaulax Catenella under artificial, continuous (700 ft-C) and alternating light—dark illumination (700 ft-C; 11 hrs. light—13 hrs. dark)

(fig. 8). The subcultures from pond study 1 (low salinity, 24.3‰) exhibited slightly greater growth than the subcultures from pond study 2 (high salinity, 34.5‰) under both continuous and alternating illumination.

The maximum *G. catenella* population densities of the subcultures initially exposed to continuous illumination were lower than those usually reached in unialgal *G. catenella* cultures maintained under similar cultural conditions. This may reflect the influence of competition exerted on the *G. catenella* populations by the contaminants present in the pond culture media.

Because of poor temperature control, (i.e., subcultures receiving alternating illumination were maintained at approximately 21.5° C. during the light phase and 15° C. during the dark phase) the results of this study are equivocal. However, the possible limiting effect of short duration, low intensity illumination was not ignored.

OUTDOOR POND STUDIES

Four outdoor pond studies were conducted to study the feasibility of mass culturing *G. catenella* outdoors, in enriched sea water media, utilizing natural sunlight, and controlled with regard to temperature and initial pH.

The materials and methods pertaining to the outdoor pond cultures were followed.

Under the cultural conditions summarized in table 4, *G. catenella* was not successfully cultivated outdoors.

Table 4.—Summary of cultural conditions of pond studies 1, 2, 3, and 4

	Culture temperature (° C.)	Salinity (‰) [1]	pH				Light					Air temperature (° C.)	Total precipitation (inches)
							Intensity (ft.-c.)				Duration (hr.:min.)		
			Morning	Noon	Evening	Total [2]	Morning	Noon	Evening	Total [2]			
Pond study 1:													
Mean	16. 0	25. 7	8. 55	8. 65	8. 80	8. 65	3, 300	4, 000	350	2, 800	10:46	8. 5	1. 2
Range	12. 4–19. 9	23. 9–28. 1	------	------	------	8. 05–9. 40	------	------	------	0–>5, 000	10:36–10:57	−3–18. 3	------
Pond study 2:													
Mean	15. 1	35. 7	8. 65	8. 75	8. 85	8. 70	3, 300	4, 000	350	2, 800	10:46	8. 5	1. 2
Range	9. 4–17. 4	33. 6–38. 1	------	------	------	8. 15–9. 20	------	------	------	0–>5, 000	10:36–10:57	−3–18. 3	------
Pond study 3:													
Mean	14. 5	23. 3	8. 50	8. 60	8. 80	8. 70	2, 800	3, 550	630	2, 330	11:10	14. 5	2. 5
Range	12. 5–15. 7	20. 4–28. 5	------	------	------	8. 20–9. 20	------	------	------	0–>5, 000	11:01–11:18	9. 0–20. 0	------
Pond study 4:													
Mean	14. 4	25. 9	8. 70	8. 85	8. 95	8. 80	2, 800	3, 550	630	2, 330	11:10	14. 5	2. 5
Range	12. 3–15. 7	24. 8–28. 6	------	------	------	8. 15–9. 35	------	------	------	0–>5, 000	11:01–11:18	9. 0–20. 0	------

[1] Parts per thousand.
[2] Calculated from all measurements made during each study.

Pond Studies 1 and 2

From an initial cell density of 690 cells/ml. the *G. catenella* population in pond study 1 exhibited an unusually rapid increase to 3,000 cells/ml. in 2 days. The cell count then declined erratically until it reached 0 cells/ml. on the 13th day (fig. 9).

A rapid increase in the *G. catenella* population was also observed in pond study 2. In the first 2 days, viable cell density increased from 860 cells/ml. to a maximum density of 2,400 cells/ml. before decreasing to 580 cells/ml. by the fifth day. The population density then stabilized through the remainder of the study (fig. 9).

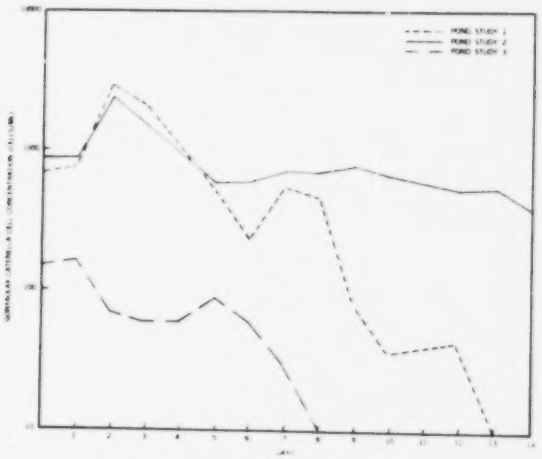

FIGURE 9.—Growth of Gonyaulax Catenella during pond studies 1, 2, and 3

The sudden increase in viable cell counts observed between the first and second days of growth in both studies was speculated to be the result of biased sampling. It was noted that core samples were being collected by inadvertently immersing the pipettes slightly faster than they were filling. This could have resulted in an excess of bottom culture being sampled. As was mentioned earlier, the ponds were not completely mixed prior to sampling; therefore, many of the cells in the inoculum had an opportunity to settle or migrate to the bottom and constitute an unrepresentative portion of the samples withdrawn in the manner described.

Although the cultural conditions of both pond studies did not support the growth of *G. catenella* they were conducive to the growth of several other organisms, primarily diatoms, occurring naturally in the sea water used for the culture media base. These organisms began blooming after 4 days in pond study 1 and after 3 days in pond study 2. Before showing signs of a decline on the 12th day in pond study 1 and the 11th day in pond study 2, the contaminants attained such high population densities that the pond bottoms could not be seen through the green-brown cultures.

Pond Studies 3 and 4

The cultural conditions of pond study 3 were short of ideal. After receiving an inoculum whose population density was low and declining, the pond's *G. catenella* population declined from an initial density of 150 cells/ml. to a density of 70 cells/ml. in 2 days. It remained at approximately this density through the sixth day during which time a large volume of chlorinated tapwater was discovered to have escaped into the culture medium through several small leaks in the heat exchange coil. These leaks were primarily responsible for the dilution of the culture medium from an initial salinity or 28.4‰ to a salinity of 20.4‰ during this period. The cell density declined to 0 cells/ml. on the eighth day.

Culture samples were collected on the third day of pond study 4 and forwarded, in a fixed (2 percent formalin) and unfixed condition, to the Florida State Board of Conservation, Marine Laboratory, St. Petersburg, Fla., for identification of the preponderant contaminants. The diatom species listed in table 5 constituted most of the contamination: however, ciliates, flagellates, and a nonmotile, unicellular green alga were also observed but were not identified (Steidinger, Personal Communication).

Table 5.—Diatom contaminants observed during pond study 4 [1]

Species	*Cells/L*
Skeletonema costatum	33,300,000
Asterionella japonica	6,340,000
Nitzschia closterium	260,000
Rhizosolenia fragilissima	110,000
Thalassiosira rotula (?)	80,000
Thalassionema nitzschioides	60,000
Coscinosira polychorda	40,000
Thalassiosira decipiens	30,000
Cerataulina pelagica	30,000
Amphiprora sp.	20,000
Melosira moniliformis (?)	20,000
Rhizosolenia setigera	150
Nitzschia longissima	100
Coscinodiscus sp.	50
Pleurosigma sp.	50

[1] Identified and enumerated by Richard P. Saunders, Florida State Board of Conservation, Marine Laboratory, St. Petersburg, Fla.

After 3 days of growth, the contaminant blooms imparted a green tint to the pond study 3 and 4 cultures. By the sixth day the cultures were characterized by a dark amber color that was retained until the 12th day after which settling and decomposition of the contaminants occurred and clearing was observed. The growth of the two most common diatoms, *Skeletonema costatum* and *Asterionella japonica* is presented in figure 10.

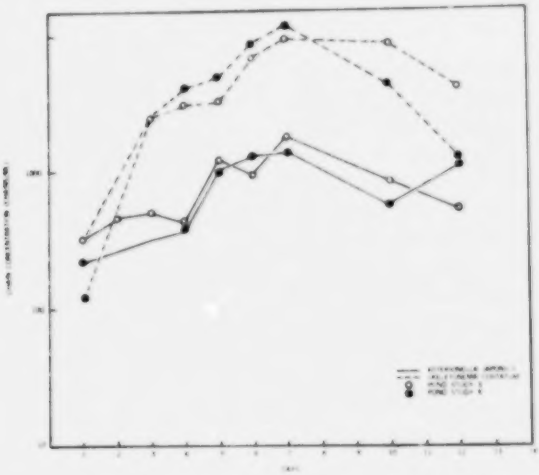

FIGURE 10.—Growth of Asterionella Japonica and Skeletonema Costatum during pond studies 3 and 4

Discussion

Except for the difference between the average salinity of pond study 1 (25.7‰) and pond study 2 (35.7‰), the cultural conditions of both studies were comparable (table 4). However, the occurrence of the complete "die-off" of the *G. catenella* population during pond study 1, but not during pond study 2, cannot, on the basis of illumination study II, be attributed to low salinity alone. During illumination study II, the low salinity subculture from pond study 1, exposed to continuous, low intensity illumination, reached a peak population density of 3,900 cells/ml. as compared to the maximum density of 2,100 cells/ml. reached by the high salinity subculture from pond study 2 under exactly the same cultural conditions. Even under the alternating light-dark low intensity light conditions of this experiment, low density *G. catenella* populations were maintained for 13 days in both pond subcultures without the occurrence of a complete "die-off" (fig. 8).

The combination of these results and the results of illumination study I, indicate that alternating light-dark illumination, characterized by light cycles of relatively short duration and of relatively high intensity, may have been primarily responsible for the failure to successfully cultivate *G. catenella* outdoors. However, these conditions of illumination do appear to support very low density populations of *G. catenella* that are subject to complete destruction when acted upon by a combination of environmental stresses (e.g., low salinity in conjunction with competition from other microorganisms and, possibly, fluctuating culture temperatures and pH).

The maintenance of the low density *G. catenella* populations observed during this study, under what were apparently adverse conditions, parallels the maintenance of low density populations or encystment of several toxic dinoflagellates during unfavorable conditions in nature. Under favorable conditions (e.g., optimum illumination, salinity, temperature, and nutrient levels), these same organisms reach high enough concentrations to cause massive fish kills, in the case of *Gymnodinium breve* (*12*), and shellfish toxicities, in the case of *G. catenella* and *G. tamarensis* (*13*).

The relationship of illumination to shellfish toxicities occurring as a result of increased population densities of toxic photosynthetic dinoflagellates has been discussed by several investigators. Prakash and Medcof's (*13*) study of the factors affecting shellfish toxicities in the vicinity of New Brunswick, Canada, long the Atlantic coast, revealed a positive correlation between increased sunshine duration and shellfish toxicities—toxicities caused by the toxic photosynthetic dinoflagellate, *Gonyaulax tamarensis*.

In a review of the circumstances surrounding an outbreak of shellfish poisoning along the Straits of Georgia, on the Pacific coast, Anderson (*14*) noted that the sunshine duration prior to the outbreak was considerably greater than the 10-year average for the same period. The photosynthetic dinoflagellate thought to be the causative organism was not identified. However, several species of the genera *Gonyaulax*, *Gymnodinium*, and *Peridinium* (members of which are known to be toxic), were earlier identified from the same waters.

Several species of the genus *Gonyaulax* have been shown to carry out certain cell functions (i.e., photosynthesis, cell division, and luminescence), in a rhythmical manner that persist under a variety of light conditions and reappear even after being lost due to the cell's exposure to high intensity light or long periods of continuous, low intensity illumination (*15*). Nevertheless, the possibility that the *G. catenella* stocks used during this study had adapted to continuous, low intensity illumination during the many generations that had occurred since their original isolation and were unable to quickly adjust to the short duration, relatively high light intensities experienced outdoors should be considered.

During studies comparing the growth patterns of *Gonyaulax tamarensis* and *G. catenella*, Prakash (Personal Communication) found that the latter grew best at a low light intensity of 275–300 ft.-c. Based on Yentsch's (*16*) discussion of the effect of light intensity on photosynthesis, it

seems possible that the photosynthetic activity of *G. catenella* adapted to low light intensities in the laboratory would be inhibited by the higher light intensities experienced outdoors—particularly in a shallow pond where any effort to seek conditions of lower light intensity would be restricted by depth. If this were the case, and not a natural reaction, the use of *G. catenella* seed stocks recently isolated from the natural environment or adapted to the illumination conditions characteristic of the geographical location of the proposed culture site may be a prerequisite for outdoor cultivation.

Inconsistent *G. catenella* growth patterns observed in laboratory cultures, possibly resulting from the combined effects of insufficient or excessive aeration, nutritional deficiences in the culture medium, and variations in the chemical composition of the medium's sea water base, should be corrected if reliable *G. catenella* production is to be achieved in the laboratory or outdoors.

From the standpoint of reproducibility, the use of a chemically defined culture medium may be more advantageous than relying on enriched sea water as the culture medium. Such a defined medium would also eliminate or reduce the decontamination procedures required to rid the enriched sea water medium of contaminating micro-organisms that not only compete with *G. catenella* but that could hamper toxin extraction efforts. If the use of a chemically defined medium on a large scale is deemed undesirable, the quality and pretreatment (decontamination) of the sea water used for the enriched sea water medium will have to receive more attention.

From the standpoint of sea water quality, as well as other factors, the selection of a large scale culture site should be given careful consideration. It will not only be necessary to consider the proposed site's seasonal air temperatures, precipitation and, possibly, illumination characteristics, but also its proximity to a source of high quality sea water—preferably sea water of high salinity and free of agricultural and industrial wastes. The facilities required to transport large volumes of this water to the site should also be considered.

These large volumes of sea water will have to be decontaminated to eliminate or reduce the problems created by contaminant growth. Because of the difficulty of maintaining sterile conditions in exposed outdoor cultures, the substantial reduction of the contaminants would be a much more practical objective than the complete decontamination of large volumes of water by sterilization. The practicability of employing one, or a combination, of the following treatments as a means of effecting a reduction of the contaminants that approaches sterilization should be considered: (1) Heating; (2) filtration; and (3) chemical toxicants or antibiotics. The use of heat or filters would be dependent upon the practicability of scaling-up such treatments. On the other hand, the use of chemical toxicants or antibiotics would have to be based upon a well-founded understanding of their effect on *G. catenella* as well as on the contaminants. Only when these relationships were known would it be possible to select an agent and its dosage, toxic to the contaminants yet not toxic to *G. catenella*. A better appreciation of the critical nature of such a selection can be obtained from discussions of the effect of various toxicants on algae by Krauss (*17*) and Ukeles (*18*).

Carbon dioxide utilization and fixation associated with light-dependent increases and decreases in the photosynthetic activity of the overwhelming numbers of contaminants is believed to have caused the pronounced daily pH fluctuation of the outdoor pond cultures. The cumulative effect of these fluctuations was reflected by a gradual increase in pH after an initial decline. *Gonyaulax polyedra*, exposed to alternating light-dark illumination, have been shown to effect similar daily pH rhythms (*15*); therefore, unialgal cultures of *G. catenella* might be expected to produce similar pH changes. The effect pH changes had on the mixed, outdoor *G. catenella* populations cannot be determined because of the poorly defined influences of the other cultural conditions. Its effect should be studied, however, so that, if necessary, pH control measures can be designed into the outdoor cultural system.

Several refinements of the outdoor pond temperature control system could be made to increase the system's sensitivity and possibly effect sea water decontamination. These refinements are as follows:

1. Rather than relying on a master pond to control the temperature of several ponds, provide each pond with a temperature control and recording system. A control system consisting of one mercury thermoregulator and normally closed electronic relay, to which a normally open electric solenoid (chilled waterline) is connected, might provide more sensitive temperature control

than the hot and chilled water system shown in figure 3.

2. Fabricate the heat exchange coils out of a nontoxic, heat resistant material that allows more efficient heat transfer than plastic.
3. It may be possible to decontaminate the sea water in ponds constructed with nontoxic, heat resistant materials by circulating steam through the heat exchange coils until the sea water becomes hot enough, over a sufficient period of time, to kill most of the contaminants. The steam generator required for such an operation could be used during the normal culture temperature control operation as well as to decontaminate the entire culture system at the end of the *G. catenella* cultivation period, thereby eliminating the possibility of contaminating the pond systems with toxicants during *G. catenella* decontamination.

Many of the cultural problems associated with the mass outdoor cultivation of *G. catenella* are common to the mass cultivation of several other photosynthetic organisms. A number of these problems have already been solved for some of these forms; others remain unsolved. An understanding of earlier approaches to mass cultivation, some of which have been reviewed by Tamiya (*19*) and Krauss (*20*), may provide some insight to, as yet, undefined problems as well as provide solutions to some of the immediate problems related to the mass, outdoor cultivation of *G. catenella*.

Recommendations

Although mass, outdoor cultivation of *G. catenella* was not achieved during this study, this, by no means, indicates that outdoor cultivation would not be feasible under the proper conditions. The scope of inquiry of this study was such that these proper conditions could not be adequately defined; however, several problem areas related to them were exposed. A better understanding of these problem areas should be obtained if evaluations of the feasibility of outdoor cultivation are to be pursued.

The major areas that should be studied before large scale, outdoor feasibility studies are again initiated are as follows:

1. Effects of alternating light-dark cycles of illumination on growth of *G. catenella* with emphasis oriented toward illumination intensity-duration relationships.
2. Methods of reducing or eliminating contaminants from the culture medium.
3. Effects of sea water and culture medium composition on *G. catenella* growth with emphasis on sea water quality.
4. Effects of aeration and fluctuating pH on *G. catenella* growth with emphasis on determining whether control is necessary.
5. Studies oriented toward selecting strains of *G. catenella* that exhibit growth characteristics conducive to outdoor cultivation.
6. Need and limits of temperature control for outdoor cultivation of *G. catenella*.

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